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SYMBIOTIC ASSOCIATION OF *PHOTOBACTERIUM FISCHERI* WITH THE MARINE LUMINOUS FISH *MONOCENTRIS JAPONICA:* A MODEL OF SYMBIOSIS BASED ON BACTERIAL STUDIES

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Mutually beneficial symbioses involving procaryotes and multicellular eucaryotes have been studied in systems such as the rumen (Gall and Huhtanen, 1951; Hungate, 1963), the root nodule of legumes (Allen and Allen, 1950), and the digestive tract of arthropods (Brooks, 1963; Fogelsong, Walker, Puffer and Markovetz, 1975). Such associates of bilateral benefit are often termed mutualisms (Henry, 1966).

The occurrence of luminous bacteria in specialized light-emitting organs of a variety of marine fishes is another example of procaryote/eucaryote mutualism. The fish provides the luminous bacteria within its light organ with a sheltered environment and a supply of nutrients and oxygen. The bacteria in turn serve as a continuous source of light which the fish uses for a variety of purposes (Harvey, 1952). The importance of this luminescence in the behavior of the flashlight fish, *Photoblepharon palpebratus,* has been described by Morin, Harrington, Kreiger, Baldwin and Hastings (1975) and McCosker and Lagios (1975). Although anatomical and histological morphology of the symbiotic light organs of a number of these fishes has been studied (Harvey, 1952; Ahrens, 1965), little is known of the biochemical interactions inherent in these interspecies associations.

Of the four species of luminous bacteria (Reichelt and Baumann, 1973), only two have been previously reported as symbionts in the light organs of fishes. In this report a third species, *Photobacterium fischeri* is identified as the bacterial component of the light organ of the Japanese pinecone fish, *Monocentris japonica.* Thus, for the first time a symbiotic niche has been found for this species. A representative bacterial isolate from the light organ is characterized with regard to physiological parameters of its light emitting system and a speculative model of the symbiosis discussed.

Materials and Methods

Bacterial strains and media

Luminous bacteria from the light organ of *Monocentris japonica* were isolated as described below. Additional strains used were *Photobacterium fischeri* (B-398) and *Beneckea harveyi* (B-392), a strain previously designated *P. fischeri* MAV (Nealson and Markovitz, 1970; Nealson, Eberhard and Hastings, 1972). The strain numbers refer to those assigned by Reichelt and Baumann (1973). The generic assignment of some species is not yet agreed upon. Hendrie, Hodgkiss

and Shewan (1970) proposed four groups: *Photobacterium phosphoreum, P. mandapamensis, Vibrio fischeri,* and *Lucibacterium harveyi.* Reichelt and Baumann (1973, 1975), whose assignments we follow, referred to these groups as *P. phosphoreum, P. leiognathi, P. fischeri,* and *Benecka harveyi,* respectively.

The sea water media used in this study were prepared with artificial sea water (ASW) consisting of 0.4 M NaCl, 0.1 M $MgSO_4$ $7H_2O$, 0.02 M KCl, and 0.02 M $CaCl_2$ $2H_2O$ (MacLeod, 1968). The basal medium broth (BM) contained 50 mM Tris-HCl (pH 7.5), 19 mM NH_4Cl, 0.33 mM K_2HPO_4 $3H_2O$, 0.01 mM $FeSO_4$ and half-strength ASW (MacLeod, 1968). Basal medium agar (BMA) was prepared by separately sterilizing and then mixing equal volumes of double-strength BM and 20 g of Difco Noble Agar per liter. Compounds serving as sole sources of carbon and energy were filter-sterilized (0.2 μ Nucleopore) and added to the already autoclaved medium. For a complex medium broth (LM), or agar (LMA) 5 g Bacto-Peptone and 3 g Difco Yeast Extract and 3 ml glycerol were added to the recipe for BM. All media used in experiments dealing with acid production were modified by replacement of Tris buffer with 50 mM Hepes (pH 7.5) and by the exclusion of glycerol. Various sugars were added as indicated.

Living specimens of the Japanese pinecone fish, *Monocentris japonica,* were collected in the summer of 1975, fifty miles southeast of Tokyo and shipped alive to the Steinhart Aquarium. Light organs from four fish (A–D) were used. Bacterial isolation (A) was the only one performed on a healthy living fish. The other three isolations (B, C and D) were made from intact light organs of fish that had been dead for less than 12 hours prior to sampling. Otherwise the procedure was the same for the four isolations. The lower jaw containing the pair of anterio-lateral light organs was placed on ice and the surface of the organ and surrounding tissue was swabbed with 75% ethanol. The organ was then slit with a sterile razor blade and a sterile micropipette inserted into the organ matrix from which about 2–5 μl of organ fluid could be removed. This fluid was diluted into sterile sea water by a factor of 5×10^6 and several 0.1 ml aliquots of the diluted fluid were spread on LMA plates which were incubated at 18° C.

Methods

Taxonomic identification of the luminous bacterial isolates was accomplished using criteria established by Reichelt and Baumann (1973). The method involves a determination of nutritional versatility on minimal medium (BMA) with one of twelve compounds as sole source of carbon and energy. In addition, the production of three extra-cellular enzymes was monitored as well as the ability to grow at 35° C on LMA. These sixteen characteristics are diagnostic for the four species of luminous bacteria, *Beneckea harveyi, Photobacterium fischeri, P. phosphoreum,* and *P. leiognathi* (Reichelt and Baumann, 1973). It should be noted here that the species of *Photobacterium* referred to as *P. mandapamensis* by Reichelt and Baumann (1973) has been named, on the basis of priority as *P. leiognathi* (Reichelt and Baumann, 1975). The table also contains data concerning the production of a yellow pigment, and the type of decay kinetics of *in vitro* luciferase assays (Hastings and Mitchell, 1971). These additional tests

have recently been added to the diagnostic taxonomy of the luminous bacteria (J. Reichelt, personal communication).

Growth of batch cultures was monitored both by optical density at 660 nm in a Coleman Jr. II Spectrophotometer, and by electronic counting using a Coulter ZBI Particle Counter. An optical density value of 0.1 units is equivalent to 2.7×10^8 cells/ml for a range of 0.05–0.5 optical density units.

Cells in liquid culture were monitored for light production utilizing an EMI Type 9781A phototube and Pacific Photometrics model 110 amplifier with an Esterline Angus Servo Speed recorder. Periodically a 0.1 ml sample of the culture was removed to a clean glass scintillation vial. The vial was placed in a light-tight chamber and exposed to the phototube to measure the level of *in vivo* luminescence. The output of the photometer was expressed in light units, where one light unit was determined to be 2×10^{10} quanta/sec by the radioactive standard of Hastings and Weber (1963).

Autoinducer (Nealson, Platt and Hastings, 1970), which accumulates in the culture medium, was prepared by growing a representative strain of *Monocentris* symbiont (MJ1) in BM to an optical density value of 0.8 (3.5×10^9 cells/ml). Cells were removed from the spent growth medium by centrifugation and the supernatant fraction was sterilized by filtration (Nucleopore, 0.2 μ). The autoinducer fraction could then be frozen until assayed. A cross reaction was prepared to determine how addition of the autoinducer preparation affected the onset of bioluminescence in strains MJ1, and B-398. Cells of these strains were innoculated to a low optical density (0.01) in 20 ml of BM. Five ml were dispensed to two growth tubes, and 0.1 ml of the autoinducer preparation added to one tube. The second tube was a control receiving 0.1 ml of BM. The tubes were shaken at 150 rpm and 23° C, and growth and luminescence monitored at 15 minute intervals. Sensitivity of a strain to the presence of the autoinducer compound present in the spent medium of MJ1 was indicated by a significantly earlier onset of induction of luminescence in tubes with added inducer, compared to the control tubes (Eberhard, 1971).

The glucose concentration in cell-free medium was determined by the glucose oxidase reaction using the Glucostat method (Worthington Biochemical). Pyruvate was assayed in a cuvette containing 2 ml of 50 mM Tris buffer (pH 7.5), 0.2 ml of 10 mM NADH and 0.1 ml of medium sample. The absorbance at 340 nm was measured using a Beckman DU spectrophotometer and the decrease in absorbance after addition of 2 units of LDH (0.1 ml) was recorded (Lowry and Passonneau, 1972). This value was compared to a standard curve using known concentrations of pyruvic acid.

Cells were innoclulated into 250 ml flasks containing 150, 100, or 50 ml of LM. The flasks were placed on a New Brunswick G24 Environmental Incubator rotary shaker at 100 rpm and 21° C. Because of the gentle shaking, oxygen diffuses more slowly into medium in the flask with the lower surface to volume ratio (150 ml) than into the flask with 100 ml, which in turn is slower than the flask with 50 ml. All of the cultures have a characteristic aerobic growth rate up to an optical density of 0.15 to 0.25. After this point the growth rate is limited to an extent dependent on the degree of oxygen availability. Thus the effect of oxygen limitation on the development of the luminescence system can be ascertained.

Tubes containing 5 ml of modified LM were innoculated with log-phase cells of MJl to a concentration of 10^7 cells per ml. Sterile solutions of either glucose, mannitol, glycerol, galactose or mannose were added to pairs of tubes to give a 0.2% solution and the cultures grown at 22° C and 200 rpm. At an optical density of 0.3 (4×10^8 cells/ml) the cells were harvested by centrifugation (13,000 *g* for 15 min). Organic acids in the supernatant were detected by the gas-liquid chromatographic method of the Virginia Polytechnical Institute Anaerobic Laboratory (1973).

MJl cells were grown in 35 ml of modified LM plus 0.2% glucose by shaking at 150 rpm in a 200 ml flask at 22° C. At an optical density of 0.46 (1.2×10^9 cells/ml) the medium was centrifuged (10,000 *g* for 10 min) and the supernatant discarded. The pellet was resuspended in 5 ml of ice-cold, sterile sea water and recentrifuged. After carefully drawing off the supernatant, 5 ml of modified BM plus 0.2% glucose was added to the tube and the pellet rapidly resuspended. One ml was distributed to each of 5 small (10 ml capacity) centrifuge tubes which were immediately placed on a shaker at 300 rpm and 22° C. At 0, 5, 10, 15 and 20 minutes, a tube was removed from the shaker and plunged

TABLE I

Results of the taxonomic characterization of 48 bacterial isolates obtained from the luminous organs of four fish (A, B, C and D) compared to the phenotype of the standard strain of Photobacterium fischeri *(B-398). Columns summarize all phenotypes observed, plus (+) denoting presence of trait, and minus (−) denoting absence. Kinetics of* in vitro *luciferase assay were fast-type (F) or slow-type (S). (P.f. represents* Photobacterium fischeri; *B. sp.,* Beneckea *species.)*

Number of isolates from each fish	*Photobacterium fischeri* (B-398)	Fish									
		A/29		B/5		C/5	D/9				
Tests											
Growth on:											
Cellobiose	+	+	+	+	+	+	+	+			
Maltose	+	+	+	+	+	+	+	+			
d-Xylose	−	−	−	−	−	−	−	−			
Mannitol	+	+	+	−	−	+	−	+			
d-Gluconate	−	−	−	−	−	−	−	+			
d-Glucuronate	−	−	−	−	−	−	−	+			
dL-Lactate	−	−	−	−	−	−	−	+			
Acetate	+	+	+	+	−	−	+	+			
Pyruvate	−	+	+	+	+	+	+	+			
Propionate	−	−	−	−	−	−	−	+			
d-α Alanine	−	−	−	−	−	−	−	+			
L-Proline	+	+	+	−	−	+	+	+			
Extracellular production of:											
Amylase	−	−	−	−	−	+	−	+			
Gelatinase	−	−	+	−	−	−	−	+			
Lipase	+	+	+	+	+	+	+	+			
Growth at 35°C	±	+	+	−	−	−	−	+			
Produce yellow pigment	+	+	+	+	+	+	+	−			
Luciferase kinetics	F	F	F	F	F	F	F	S			
Number of isolates with phenotype		27	2	4	1	5	8	1			
Taxonomic identity	*P.f.*	*P.f.*	*P.f.*	*P.f.*	*P.f.*	*P.f.*	*P.f.*	*B. sp.*			

into ice water to suspend cellular activity. The tubes were centrifuged and the supernatant examined for glucose and pyruvate by enzyme assays.

Results

Isolation and taxonomy

The light organ of *Monocentris japonica* contains symbiotic bacteria that are located extracellularly in parallel tubular ducts whose possible communication with the exterior has not been described. Electron microscopy reveals these tubules to be densely packed with bacteria of a single morphological type. It is thus not surprising that cell densities of 5.6 and 9.4×10^9 bacteria per ml of organ fluid were determined from LMA plate counts of two such organs. Primary isolation of several hundred bacteria from light organs of four fish revealed that 100% of the colony forming units were luminous.

Five to twenty-nine colonies were chosen at random as representatives of the populations of four organs of four separate fish, subjected to taxonomic analysis and compared to previously identified strains (Table I). Three important results were obtained from this work: first, with the exception of a single isolate from fish "D" all isolates were of the species *Photobacterium fischeri;* secondly, isolates from each organ were predominantly of one phenotype; and thirdly, the phenotypes of bacteria from organs of different fish differed by several traits and no two were the same. It is important to note that the degree of variation of any of the six phenotypes from that of the standard strain is well within limits of positive identification as *P. fischeri.*

Bacterial physiology

It was of interest to examine in batch culture the response of *in vivo* bioluminescence of MJl cells to physiological conditions of the medium. A major factor which controls the development of luminescence is autoinducer (Nealson, Platt and Hastings, 1970; Eberhard, 1971), which is produced by luminous bacteria, excreted into the medium during growth and, upon reaching a critical concentration, induces the synthesis of luciferase. Addition of spent medium of the *Monocentris* symbiont (MJl) stimulates the induction of *in vivo* light emission of cells both of its own strain and of another strain of *Photobacterium fischeri* (B-398) (Fig. 1). This effect has been shown to be due to the presence of large amounts of autoinducer in the spent medium of MJl. As previously reported there was no cross reaction between species (Magner, Eberhard and Nealson, 1972). No effect of the MJl autoinducer on the light emission of cells of *Beneckea harveyi* was observed, nor did MJl cells induce luminescence sooner with the addition of spent medium of *B. harveyi.*

Because oxygen is a substrate for the luminescence reaction, its effect on the development of the light emitting system was examined. It can be seen that the amount of light produced per unit cell material increases when cells are grown in lower ambient oxygen concentrations (see Materials and Methods). That is, more synthesis of the luminous system occurs in cells grown in lower oxygen concentrations (Fig. 2).

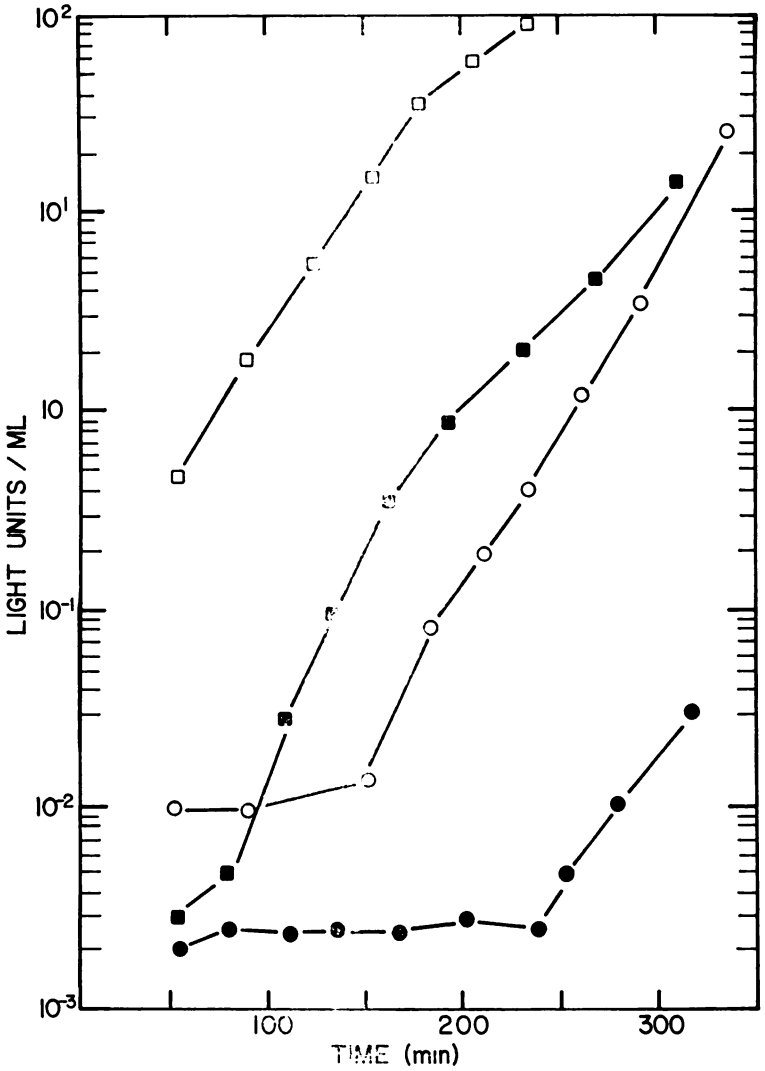

FIGURE 1. Effect of autoinducer from strain MJ1 on the induction of bioluminescence of MJ1 (open square) and B-398 (closed square). Control cultures of MJ1 (open circle) and B-398 (closed circle) received no added inducer. There was no difference in the growth rates of cultures with or without added inducer.

In addition to the factors mentioned above, the nature of the substrate utilized during growth also plays a role in the control of luciferase production. If brightly luminescing (induced) cultures of MJ1 are diluted to an optical density below 0.05 (1.4×10^8 cells/ml), the *in vivo* light of the culture will not increase until the cells have reached a certain density in the medium and luminescence is induced. If the cells are grown in a glycerol medium and diluted into fresh medium con-

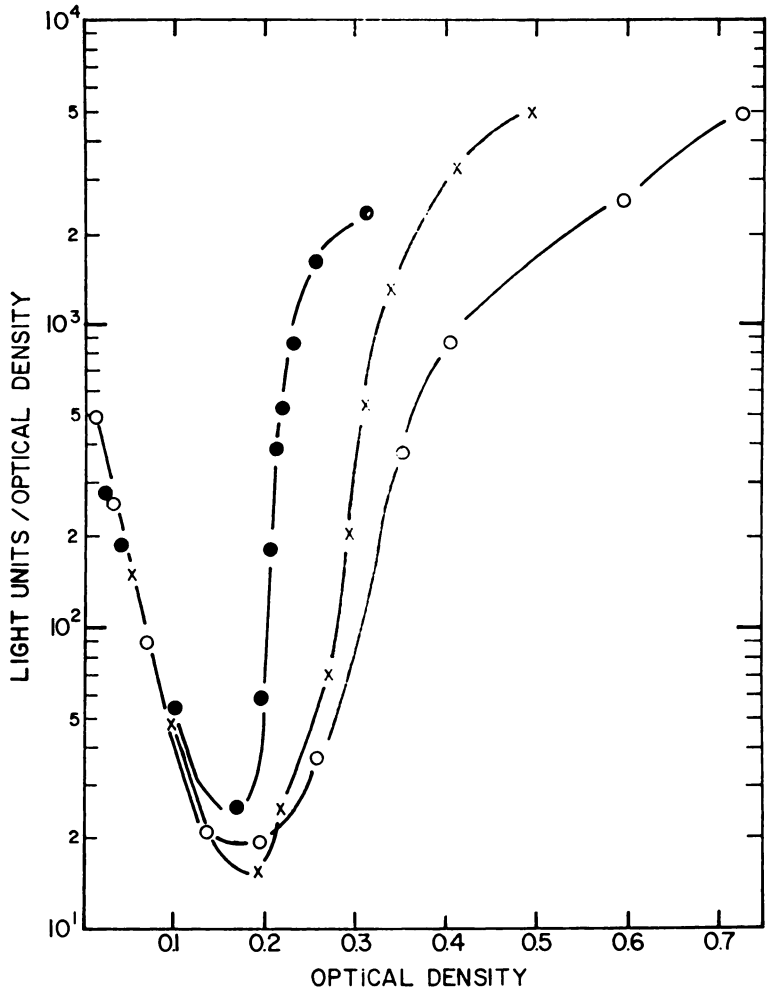

FIGURE 2. Specific activity of *in vivo* light production by MJ1 during growth at different oxygen concentrations (achieved by using different volumes of medium). Symbols designate flasks with 50 ml (open circle), 100 ml (X), or 150 ml (closed circle) of medium per flask. High values of bioluminescence per cell are reached earliest in the culture with the most volume, which is most limited for oxygen (closed circle), and latest in the culture least limited (open circle).

taining glucose, the point at which induction occurs is delayed relative to that in cells diluted into fresh glycerol medium (Fig. 3A). However, cells pregrown several generations on glucose will not experience that increased lag period when again diluted into glucose medium (Fig. 3B).

Further examination of this glucose effect reveals that glucose addition to cells growing on glycerol will either delay induction or, if induction has already begun, cause a temporary suspension of it (Fig. 4). This glucose effect is

neither reversed by cAMP (cyclic adenoside monophosphate) nor caused by the glucose analogue 2-deoxy-glucose.

During aerobic growth of MJ1 on glucose, the pH of the medium drops to below 5.0 at cell densities above 5×10^8 cells/ml and luminescence is then extinguished. Addition of strong buffers (Tris or Hepes) delays or reverses this effect, indicating that it is probably due to acid production.

To determine the identity of the acid(s) responsible for the decrease in pH of the medium, MJ1 cells were grown in a complete medium with glucose to an optical density of 0.3 (9×10^8 cells/ml). Cells were removed by centrifugation and a chromatographic analysis was performed on extracts of the medium. Of the acids detectable by gas-liquid chromatography (formate, pyruvate, lactate, oxalacetate, and succinate), pyruvate was the principal compound present in the spent medium, sometimes reaching concentrations of several millimolar. In addition, pyruvate concentration is a direct function of cell number. Growth on galactose, mannose, glycerol or mannitol, however, results in less than 5% of the acid levels obtained with glucose.

To determine what percentage of the glucose utilized by the cells was being converted to pyruvate cultures growing in a complete medium with glucose were transferred to minimal medium with glucose. Both the utilization of glucose and the excretion of pyruvate were then monitored for twenty minutes (Fig. 5). Pyruvate accounted for 30–40% of the glucose-carbon metabolized based on the

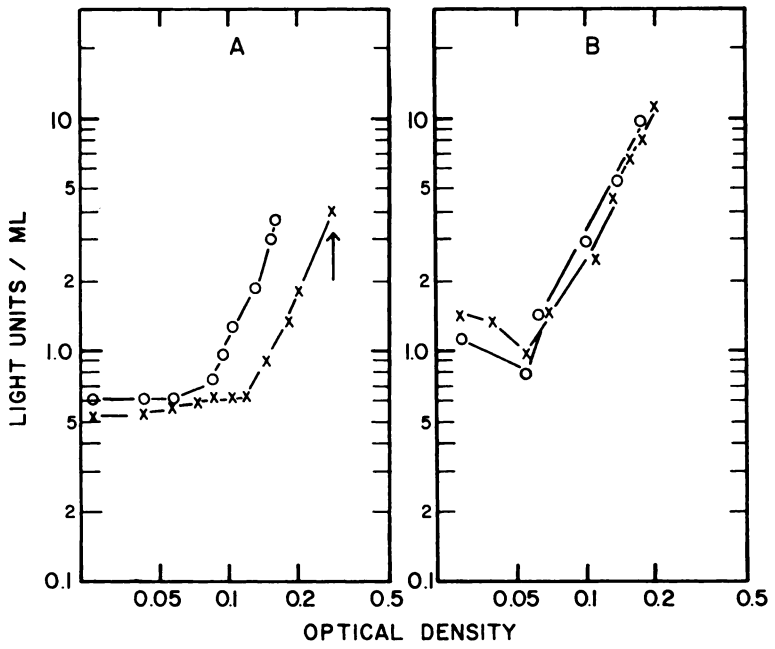

FIGURE 3. A. Development of *in vivo* luminescence in MJ1 cells pregrown in glycerol medium and innoculated into fresh medium with either glycerol (open circle) or glucose (X) as the energy source. B. Cells removed from glucose culture at arrow and innoculated into fresh medium with either glycerol (open circle) or glucose (X).

calculation [millimoles of pyruvate produced/2 (millimoles of glucose utilized)] × 100%. It should be noted that MJ1 cells are capable of metabolizing pyruvate as evidenced by their ability to grow on pyruvate as the sole source of carbon and energy (Table I).

Discussion

Although luminous bacteria are known to be the source of light for many luminous marine fishes, these bacteria have been isolated from other habitats, including the surfaces of decaying marine organisms and directly from sea water (Harvey, 1952). All four bacterial species (*Beneckea harveyi, Photobacterium fischeri, P. phosphoreum,* and *P. leiognathi*) have been isolated both directly from sea water or as saphophytes; however, only members of the genus *Photobacterium* have been found in symbiotic association. Such findings have led to a descriptive designation of *Beneckea harveyi* as "free-living" and the other genus of luminous

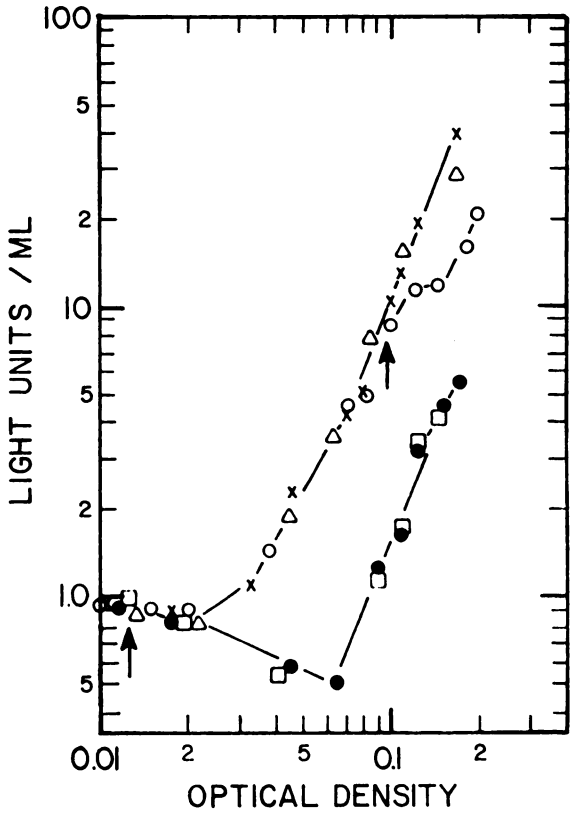

Figure 4. Effect of the addition of glucose on *in vivo* induction of the luminous system of MJ1: glycerol control (no glucose) (X); 0.2% glucose added at first arrow (closed circle); 0.2% glucose plus 0.3 mg/ml cAMP added at first arrow (open square); 0.2% glucose added at second arrow (open circle); and 0.2% 2-deoxyglucose at second arrow (open triangle.)

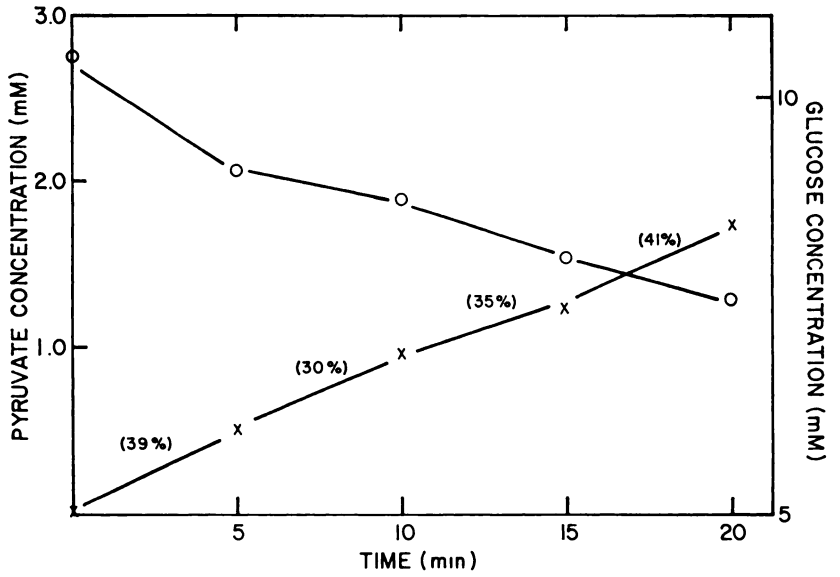

FIGURE 5. Appearance of excreted pyruvate (X) and removal of glucose (open circle) during short term exposure of strain MJ1 to minimal medium plus glucose (3.5×10^9 cell/ml). Numbers in parentheses indicate the percentage of glucose-carbon utilized that appears in the medium as pyruvate.

bacteria, *Photobacterium,* as "symbiotic" (Hastings and Mitchell, 1971). However, of the three species of *Photobacterium,* only *P. leiognathi* (Boisvert, Chatelain and Bassot, 1967; Reichelt and Baumann, 1975) and *P. phosphoreum* (Herring, 1975) have been found associated with light organs of luminous fishes. No report of a symbiotic niche for *P. fischeri* has yet been put forth. In this study we have shown such an association of *P. fischeri* with the luminous fish *M. japonica.*

Yasaki (1928) first established the bacterial origin of the luminosity of the Japanese pinecone fish *Monocentris japonica.* More recently Graham, Paxton and Cho (1972) characterized 13 luminous bacteria isolated from the Australian pinecone fish, *Cleidopus gloriamaris.* Although some of these were certainly *P. fischeri,* it was not reported which of the isolates were obtained directly from the organ and which were from the mouth and body surfaces of the fish. Luminous strains from the light organs of a number of other species of fishes have also been isolated and studied (Harvey, 1952; Boisvert *et al.,* 1967; Yoshiba and Haneda, 1967; Hastings and Mitchell, 1971). Although it was known from these studies that light organs contain luminous bacteria that appear similar both microscopically and in colonial morphology, these involved no taxonomic characterization. In order to understand the symbiotic relationship between fish and microbe, the identity of the bacterial component of the association must be known. In the present study bacteria were isolated only from the interior of the light organs and, of the 48 isolates from four fish, all but one clearly belong to the species *Photobacterium fischeri.* Similar findings for isolates of *P. leiognathi* from several leiognathid fishes will be reported elsewhere (Nealson, Reichelt and Hastings, in preparation).

The way in which the luminous organ obtains its bacterial innoculum is not known; nor is the degree of isolation of the organ culture from exchange and/or contamination with the external environment known. Clearly there must be host mechanisms involving selection for symbiont characters, but as yet we have no knowledge as to the specific features and mechanisms which operate.

The several physiological properties of MJ1 described in this study may be considered in terms of their suitability for adaptation to symbiosis. For example, the occurrence of autoinduction could provide a way for the bacteria to enjoy two different "life styles": symbiotic and free living. In bacteria confined within the luminous organ, the autoinducer of luciferase could accumulate and stimulate the synthesis of the bioluminescence system; whereas while free in sea water, no such accumulation would occur.

The enhancement of the synthesis of the luminescent system at low oxygen levels might also be related to the symbiotic relationship. The maintenance of a low ambient oxygen concentration in the light organ would produce maximal luminescence with a minimal commitment to bacterial growth.

It is difficult to envision a metabolic purpose for harvesting only a small amount of the energy available in glucose and excreting pyruvate, a compound which the cell is quite capable of catabolizing further. It is known, however, that excretion of metabolites is a characteristic phenomenon of symbioses in general (Buchner, 1965; Smith, Muscatine and Lewis, 1969). There are numerous examples of regulatory and nutritional communication between symbiotic partners by means of excretion of large amounts of carbon compounds (Muscatine, Karakashian and Karakashian, 1967; Muscatine, Boyle and Smith, 1974).

In *B. harveyi* the induction of the synthesis of the luminescent system is repressible by glucose, this being reversible by cAMP (Nealson, Eberhard and Hastings, 1972). In MJ1 there is no such catabolite repression of bioluminescence, as defined by Ullman and Monod (1968) and Tyler and Magasanik (1970). Although glucose exerts a temporary inhibition upon the development of luminescence, it is not reversible by cAMP, nor does it occur with the analogue, 2-deoxyglucose. Once adapted to growth on glucose, MJ1 cells can produce an amount of light equal to that obtained with glycerol. Thus, in the symbiotic light organ, *Monocentris* could supply its bacterial culture with glucose as a substrate and still achieve a high efficiency of light production with *P. fischeri* (MJ1) as the symbiont, but not with *B. harveyi.*

The blood of marine fishes contains glucose as its principal nutrient (Dittmer, 1961; Umminger, 1970); at the concentrations reported (0.07 to 0.15%), glucose could readily support the metabolic demands of these bacteria, based on studies with the isolated cells. Glucose utilization would lead to the excretion of pyruvic acid and unless this metabolite were removed, the pH within the organ would drop and extinguish the bioluminescence. We can speculate that the fish tissue surrounding the bacteria could absorb the pyruvate and metabolize it aerobically using the numerous large mitochondria located in the cells adjacent to the bacterial culture. The utilization of oxygen in this process could serve to poise its concentration at the level optimal for luciferase synthesis. Such a model could account for a system by which the fish regulates the oxygen concentration within the organ to maintain a population of slowly growing, brightly luminescing bacteria.

SUMMARY

Isolation of bacteria from the luminous organ of the fish *Monocentris japonica* has revealed that the organ contains a pure culture of luminous bacteria. For the four fish examined, all contained *Photobacterium fischeri* as their luminous bacterial symbiont. This is the first time that *P. fischeri* has been identified in a symbiotic association.

A representative isolate (MJ1) of the light organ population was selected for *in vivo* studies of its luminous system. Several physiological features suggest adaptation for symbiotic existence. First, MJ1 has been shown to produce and respond to an inducer of luciferase that could accumulate in the light organ. Secondly, the specific activity of light production was seen to be maximal under low, growth-limiting concentrations of oxygen. Thirdly, unlike another luminous species (*Beneckea harveyi*), synthesis of the light production system of these bacteria is not catabolite repressed by glucose—a possible source of nutrition in the light organ. Fourthly, when grown aerobically on glucose these bacteria excrete pyruvic acid into the medium. This production of pyruvate is a major process, accounting for 30–40% of the glucose utilized and may serve as a form of regulatory and nutritional communication with the host.

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